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# Heterogeneity of T Cell Responses to Pandemic pH1N1 Monovalent Vaccine in HIV-Infected Pregnant Women

Adriana Weinberg,<sup>1</sup> Petronella Muresan,<sup>2</sup> Kelly Richardson,<sup>1</sup> Terence Fenton,<sup>2</sup>  
Teresa Dominguez,<sup>1</sup> Anthony Bloom,<sup>3</sup> D. Heather Watts,<sup>4,\*</sup> Mark J. Abzug,<sup>1</sup>  
Sharon A. Nachman,<sup>5</sup> and Myron J. Levin,<sup>1</sup> for the P1086 Study Team

## Abstract

We investigated the Th1 protective and regulatory T and B cell (Treg and Breg) responses to pH1N1 monovalent influenza vaccine (IIV1) in HIV-infected pregnant women on combination antiretroviral therapy (cART). Peripheral blood mononuclear cells (PBMCs) from 52 study participants were cryopreserved before and after vaccination and analyzed by flow cytometry. pH1N1-specific Th1, Treg, and Breg responses were measured in PBMCs after *in vitro* stimulation with pH1N1 and control antigen. The cohort analysis did not detect changes in pH1N1-Th1, Treg, or Breg subsets postvaccination. However, individual analyses distinguished subjects who mounted vigorous Th1 responses postvaccination from others who did not. Postvaccination, high pH1N1-Th1 correlated with high pH1N1-Treg and Breg responses, suggesting that low influenza effector responses did not result from excessive vaccine-induced immune regulation. High postvaccination pH1N1-Th1 responses correlated with baseline high PHA- and pH1N1-IFN- $\gamma$  ELISpot and circulating CD4<sup>+</sup>CD39<sup>+</sup>% and CD8<sup>+</sup>CD39<sup>+</sup>% Treg, with low CD8<sup>+</sup> cell numbers and CD19<sup>+</sup>FOXP3<sup>+</sup>% Breg, but not with CD4<sup>+</sup> cell numbers or HIV viral load. These data highlight the heterogeneity of T cell responses to vaccines in HIV-infected individuals on cART. Predictors of robust Th1 responses to IIV include CD8<sup>+</sup> cell numbers, T cell functionality, and circulating Breg and Treg.

## Introduction

INFLUENZA INFECTIONS ARE FREQUENT and have increased morbidity in HIV-infected children and adults, including pregnant women, which underscores the importance of vaccine-conferred protection. Multiple studies showed poor antibody responses to influenza vaccines in HIV-infected individuals.<sup>1–4</sup> However, studies also showed the efficacy of trivalent inactivated seasonal influenza vaccines (IIV3) in HIV-infected adults including pregnant women.<sup>3,4</sup>

This implies that antibody titers against influenza measured by hemagglutination inhibition (HAI) may not be a good surrogate of protection in HIV-infected individuals. HAI titers  $\geq 1:40$  were shown to decrease the incidence of influenza disease in immune-competent young adults by 50%, and the ability of IIVs to generate HAI titers  $\geq 40$  has

been used as a benchmark to predict vaccine efficacy and to ensure FDA approval of new IIV products. Although a majority of studies conducted in immune-competent IIV recipients supports the value of the HAI  $\geq 40$  standard as a predictor of protection, recent studies have disputed the validity of this HAI standard both in immune-competent children and adults.<sup>5–7</sup> Some potential explanations for the apparent lack of surrogacy of HAI titers in HIV-infected individuals include the following: (1) HAI-measured antibodies may not constitute a mechanistic surrogate of protection and their ability to predict protection against influenza is highly dependent on host factors, such as immunologic competency or age,<sup>8</sup> and (2) HIV-infected individuals may be highly heterogeneous with respect to immune responses due to varying degrees of immune deficiency that may not be totally reflected by CD4 cell

<sup>1</sup>University of Colorado Anschutz Medical Campus, Aurora, Colorado.

<sup>2</sup>Statistical and Data Analysis Center, Center for Biostatistics in AIDS Research, Harvard School of Public Health, Boston, Massachusetts.

<sup>3</sup>Frontier Science and Technology Research Foundation, Buffalo, New York.

<sup>4</sup>Maternal and Pediatric Infectious Disease Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, Maryland.

<sup>5</sup>State University of New York Health Science Center at Stony Brook, Stony Brook, New York.

\*Current affiliation: Office of the U.S. Global AIDS Coordinator, U.S. Department of State, Washington, District of Columbia.

numbers, HIV plasma RNA levels, or use of combination antiretroviral therapy (cART).

P1086 was a study of the International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) network that investigated the safety and immunogenicity of two double doses of the pandemic H1N1 (pH1N1) IIV1 in HIV-infected pregnant women on cART. The primary analysis of P1086 showed that the immunization regimen was safe, but the immunogenicity measured by HAI titers was lower compared with historical immune-competent adult vaccinees.<sup>9</sup> In a subsequent study of the B and T cell responses to pH1N1 in P1086 participants, we found that only pH1N1 HAI titers and IgG memory B cells increased after vaccination, whereas interferon gamma (IFN- $\gamma$ ) ELISpot-measured effector T cells (Teff) decreased, and IgA memory B cells and granzyme B (GrB) Teff did not significantly change from prevaccination to postvaccination.<sup>10</sup> Surprisingly, however, the B and T cell responses to pH1N1 vaccine in P1086 participants were generally positively correlated despite their median trajectories over time going in different directions. This observation led us to hypothesize that some important interrelationships in immune responses to pH1N1 IIV1 may be identified by correlation analyses, even in cases in which summary statistics did not indicate significant or consistent changes in group medians.

In this exploratory study, we expanded the immunogenicity analysis of the pH1N1 IIV1 in a subset of HIV-infected pregnant women from P1086 by evaluating pH1N1-specific Th1 and cytotoxic responses and regulatory responses to vaccination. Th1 and cytotoxic cell-mediated immunity (CMI) is generally protective against viral infections and both human and animal studies showed its protective role against influenza.<sup>11–19</sup>

## Participants and Methods

### Study design

HIV-infected women 18 to 39 years of age, 14 to 34 weeks gestation, and on antiretroviral therapy, who consented to this study as per local IRB stipulations, received two 30  $\mu$ g doses of unadjuvanted, inactivated pH1N1 vaccine, 21 to 28 days apart, at 31 U.S. IMPAACT sites, as previously described.<sup>9</sup> Serum, plasma, and peripheral blood mononuclear cells (PBMCs) were collected and cryopreserved at entry before administration of the first dose of vaccine, before administration of the second dose (21 to 28 days postdose 1), and 10 to 14 days postdose 2. PBMCs were collected at entry and after each vaccination on the first half of participants enrolled at sites that were certified to cryopreserve PBMCs through the Immunology Quality Assurance program of the Division of AIDS of the National Institute of Allergy and Infectious Diseases. Here we report on the baseline and postdose 1 CMI responses.

### Flow cytometry

PBMCs were frozen, stored, shipped, and thawed as per the IMPAACT version of the HIV/AIDS Network Coordination protocol ([www.hanc.info/labs/Pages/SOPs.aspx](http://www.hanc.info/labs/Pages/SOPs.aspx)). After overnight rest, PBMCs with viability  $\geq 70\%$  and viable recovery  $\geq 50\%$  were resuspended at  $10^6$  PBMC/ml in RPMI 1640 supplemented with antibiotics and 10% human AB blood group serum and were stimulated for 48 h with 2

TCID<sub>50</sub>/cell of A/California/7/2009 Pandemic X-179A H1N1 Influenza virus or medium control in the presence of 1  $\mu$ g/ml of each of anti-CD28 (BD Biosciences; L293) and anti-CD49d (BD Biosciences; B7651) monoclonal antibodies (mAbs). Brefeldin A (Sigma-Aldrich) was added to a final concentration of 10  $\mu$ g/ml for the last 12–15 h of the incubation. After washing and counting, PBMCs were surface stained using the following conjugated mAbs: anti-CD3-PECy7, anti-CD8-APC-AF 750, and anti-CD19-PECy5, and then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with anti-IL-10-APC, anti-FOXP3-FITC, anti-TGF- $\beta$ -PE (Cederlane; TB21), anti-MIP1 $\beta$ -PE (BD Biosciences; D21-1351), anti-TNF- $\alpha$ -PerCP Cy5.5 (Biolegend; MAb11), anti-Perforin-APC (Biolegend; dG9), and anti-IL-2-FITC (BD Biosciences; 5344.111). Total T and B cells and subpopulations were counted on Guava easyCyte 8HT (Millipore) and analyzed with FlowJo (Treestar). Subsets were expressed as a percentage of the parent CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, or CD3<sup>+</sup>CD19<sup>+</sup> cell population.

### Statistical methods

This was an exploratory substudy for which no sample size calculations were performed. Baseline characteristics were summarized using descriptive measures. Changes in pH1N1-specific effector, memory, and regulatory T cell subsets from baseline to postimmunization were assessed using the Wilcoxon matched pairs signed-rank tests. The final flow cytometric analyses were restricted to samples with  $\geq 100$  events in the CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> anchor gates. A sensitivity analysis showed that the inclusion of samples with  $< 100$  events in the anchor gates would not have changed the results. Spearman correlation analyses were performed to assess the strength of associations and test their statistical significance. All analyses were performed using SAS Version 9.2 (SAS Institute Inc.) and graphs were produced using the R software.

## Results

### Characteristics of the study population

This study used PBMCs from 52 subjects enrolled in the P1086 parent study who completed vaccination before delivery and had cryopreserved PBMCs stored before and after vaccination. At enrollment, women had a mean age of 27 years, and medians of 33% CD4<sup>+</sup> T cells and 1.9 log<sub>10</sub> HIV RNA copies/ml of plasma, all of which were similar to the baseline measures of the total population in the parent study (Table 1).

### Th1 and cytotoxic CMI responses to pH1N1 monovalent vaccine measured by flow cytometry

pH1N1-specific Th1 responses were characterized by the expression of interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF- $\alpha$ ), and cytotoxic responses by expression of perforin in pH1N1-stimulated PBMCs after subtraction of mock-stimulated controls. pH1N1-specific MIP1 $\beta$  expression, which is an integral component of both Th1 and cytotoxic responses, was also measured on stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In aggregate, there were no significant changes from baseline to postdose 1 in the pH1N1-specific T cell immunity (Table 2 and Supplementary Table S1; Supplementary Data

TABLE 1. DEMOGRAPHICS AND HIV DISEASE CHARACTERISTICS

Characteristic	All	Substudy
Number of subjects	119	52
Race		
Black	71 (60%)	32 (62%)
Ethnicity		
Latino	41 (34%)	21 (40%)
Age (years)		
Median	29	27
Interquartile range (IQR)	(22, 32)	(23, 31)
Gestational age (weeks)		
Median	25	27
IQR	(20, 29)	(21, 30)
Receiving ARVs	119 (100%)	52 (100%)
Type of ARV regimen		
HAART	112 (94%)	46 (88%)
Other	7 (6%)	6 (12%)
CD4 percent		
Median	32	33
IQR	(23, 39)	(27, 41)
CD4 count (cells/mm <sup>3</sup> )		
Median	481	496
IQR	(350, 647)	(388, 635)
CD8 percent		
Median	46	44
IQR	(38, 52)	(38, 51)
CD8 count (cells/mm <sup>3</sup> )		
Median	686	643
IQR	(529, 893)	(527, 851)
N	N = 109	N = 49
log <sub>10</sub> RNA count		
Median <sup>a</sup>	1.9	1.9
IQR	(1.7, 2.6)	(1.7, 2.6)

<sup>a</sup>The lower limit of detection varied among subjects depending on the assay used at the clinical research site; RNA values below the limit of detection were replaced with the lower detection limit of the assay.

TABLE 2. CHANGE IN pH1N1-SPECIFIC Th1 AND CYTOTOXIC T CELL SUBSETS FROM BASELINE TO POSTDOSE 1 pH1N1 VACCINE

Variable (%) <sup>a,b</sup>	Median (IQR)	p value <sup>c</sup>
CD4 <sup>+</sup> IL-2 <sup>+</sup>	-0.17 (-1.98, 2.02)	0.93
CD4 <sup>+</sup> MIP1 $\beta$ <sup>+</sup>	0.53 (-2.05, 2.38)	0.81
CD4 <sup>+</sup> MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	-0.09 (-0.46, 0.49)	0.91
CD4 <sup>+</sup> PERF <sup>+</sup>	-0.12 (-2.85, 2.21)	0.79
CD4 <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	-0.56 (-2.27, 1.21)	0.31
CD4 <sup>+</sup> PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	-0.32 (-0.76, 0.79)	0.69
CD8 <sup>+</sup> IL-2 <sup>+</sup>	0.56 (-2.31, 1.94)	0.85
CD8 <sup>+</sup> MIP1 $\beta$ <sup>+</sup>	0.58 (-2.60, 4.32)	0.35
CD8 <sup>+</sup> MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	0.14 (-0.79, 1.44)	0.53
CD8 <sup>+</sup> PERF <sup>+</sup>	-0.24 (-3.59, 3.93)	0.65
CD8 <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	-0.69 (-2.79, 2.62)	0.62
CD8 <sup>+</sup> PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	-0.37 (-1.45, 1.66)	0.77

<sup>a</sup>N = 49.

<sup>b</sup>Subsets, measured by flow cytometry, are expressed as a percentage of the parent CD4<sup>+</sup> or CD8<sup>+</sup> B cell population.

<sup>c</sup>Wilcoxon matched pairs signed-rank tests.

are available online at [www.liebertpub.com/aid](http://www.liebertpub.com/aid)). However, further analyses showed that Th1 CMI responses to vaccination were highly correlated (Table 3). This level of coordination of the protective CMI responses suggested that the differences among subjects represented a true difference in their ability to respond to the vaccine as opposed to a random finding.

#### Regulatory T cell responses to vaccination

To determine the effect that T cell regulation may play in the modulation of the protective immune response to the pH1N1 vaccine in HIV-infected pregnant women, we examined the changes from baseline to postdose 1 of multiple subsets of T and B regulatory subsets characterized by the expression of FoxP3, IL-10, and/or TGF- $\beta$  (Table 4 and Supplementary Table S2). In aggregate, we did not find significant changes in pH1N1-specific regulatory T or B cell after pH1N1 vaccination except for a small decrease in the CD4<sup>+</sup>TGF- $\beta$ <sup>+</sup> (median difference = -0.99%;  $p = 0.02$ ).

Further analyses of Treg responses revealed heterogeneity similar to that observed for the Th1 and cytotoxic T cell responses (data not shown). Surprisingly, however, when we examined the correlations between changes from baseline to postvaccination between pH1N1-specific Th1 and cytotoxic protective responses and regulatory responses (Fig. 1), we found significant positive correlations of Th1 and cytotoxic protective with regulatory responses to the vaccine, suggesting coordination instead of antagonism of these responses.

#### Baseline characteristics associated with CMI responses to pH1N1 immunization

To determine the factors that may contribute to the protective T cell responses generated by influenza vaccination, we performed correlation analyses of the baseline immunologic, virologic, and select demographic characteristics with pH1N1-specific CD4<sup>+</sup>MIP1 $\beta$ <sup>+</sup>% increases after vaccination used as a general exemplification of protective responses (Table 5). The data showed that baseline CD4<sup>+</sup> T cells and HIV plasma RNA load were not associated with pH1N1-specific CD4<sup>+</sup>MIP1 $\beta$ <sup>+</sup> changes after vaccination. However, the increase in pH1N1-specific CMI after vaccination was associated with high baseline pH1N1- and PHA-IFN- $\gamma$  spot-forming cells, high circulating CD4<sup>+</sup>CD39<sup>+</sup>% and CD8<sup>+</sup>CD39<sup>+</sup>% Treg of unknown specificity, and low circulating CD19<sup>+</sup>FOXP3<sup>+</sup>% Breg of unknown specificity.

#### Discussion

This study showed that HIV-infected pregnant women had highly heterogeneous Th1 and cytotoxic T cell responses to pH1N1 demonstrated by the wide interquartile differences, such that significant group changes from baseline were not observed. However, correlation analyses indicated that this heterogeneity did not merely represent random variation, since these analyses revealed patterns of interrelationships across the effector T cell data. The CMI response to influenza vaccines is important because it is essential for protective antibody responses<sup>11-13</sup> and it generates cytotoxic T cells that clear virally infected cells.<sup>14-19</sup> The strongest predictors of protective Th1 and cytotoxic CMI responses to pH1N1 IIV1 were high baseline IFN- $\gamma$  ELISpot results after *in vitro*

TABLE 3. CORRELATION OF pH1N1-SPECIFIC T CELL SUBSET CHANGES FROM BASELINE TO POSTDOSE 1 pH1N1 VACCINE

T cell subset (%) <sup>a,b</sup>	$\rho^c$ (p value)									
	CD4 <sup>+</sup>					CD8 <sup>+</sup>				
	MIP1 $\beta$ <sup>+</sup>	MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	PERF <sup>+</sup>	TNF- $\alpha$ <sup>+</sup>	PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	IL-2 <sup>+</sup>	MIP1 $\beta$ <sup>+</sup>	MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	PERF <sup>+</sup>	TNF- $\alpha$ <sup>+</sup> PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>
CD4 <sup>+</sup> IL-2 <sup>+</sup>	0.69 (<0.0001)	0.76 (<0.0001)	0.60 (<0.0001)	0.61 (<0.0001)	0.60 (<0.0001)	0.38 (0.007)	0.44 (0.001)	0.38 (0.008)	0.41 (0.003)	0.34 (0.02)
CD4 <sup>+</sup> MIP1 $\beta$ <sup>+</sup>	1	0.82 (<0.0001)	0.55 (<0.0001)	0.59 (<0.0001)	0.58 (<0.0001)	0.23 (0.11)	0.58 (<0.0001)	0.40 (0.004)	0.40 (0.004)	0.34 (0.02)
CD4 <sup>+</sup> MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	—	1	0.44 (0.002)	0.48 (0.001)	0.52 (0.0001)	0.34 (0.02)	0.54 (<0.0001)	0.54 (<0.0001)	0.45 (0.001)	0.29 (0.04)
CD4 <sup>+</sup> PERF <sup>+</sup>	—	—	1	0.51 (0.0002)	0.79 (0.0001)	0.17 (0.25)	0.23 (0.11)	0.18 (0.22)	0.42 (0.003)	0.35 (0.02)
CD4 <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	—	—	—	1	0.69 (<0.0001)	0.08 (0.58)	0.21 (0.16)	0.10 (0.50)	0.22 (0.12)	0.40 (0.004)
CD4 <sup>+</sup> PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	—	—	—	—	1	0.04 (0.79)	0.15 (0.30)	0.11 (0.44)	0.24 (0.10)	0.25 (0.08)
CD8 <sup>+</sup> IL-2 <sup>+</sup>	—	—	—	—	—	1	0.66 (<0.0001)	0.84 (<0.0001)	0.70 (<0.0001)	0.40 (0.004)
CD8 <sup>+</sup> MIP1 $\beta$ <sup>+</sup>	—	—	—	—	—	—	1	0.84 (<0.0001)	0.69 (<0.0001)	0.47 (0.001)
CD8 <sup>+</sup> MIP1 $\beta$ <sup>+</sup> IL-2 <sup>+</sup>	—	—	—	—	—	—	—	1	0.75 (<0.0001)	0.45 (0.001)
CD8 <sup>+</sup> PERF <sup>+</sup>	—	—	—	—	—	—	—	—	1	0.62 (<0.0001)
CD8 <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	—	—	—	—	—	—	—	—	—	1
CD8 <sup>+</sup> PERF <sup>+</sup> TNF- $\alpha$ <sup>+</sup>	—	—	—	—	—	—	—	—	—	1

<sup>a</sup>N=49.<sup>b</sup>Subsets, measured by flow cytometry, are expressed as a percentage of the parent CD4<sup>+</sup> or CD8<sup>+</sup> T cell population.<sup>c</sup>Spearman correlation coefficients.*Bold* represents the values that were significant at a 0.05 level. *Italics* indicates the values that were marginally significant.

TABLE 4. CHANGE OF pH1N1-SPECIFIC REGULATORY T AND B CELL SUBSETS FROM BASELINE TO POSTDOSE 1 pH1N1 VACCINE

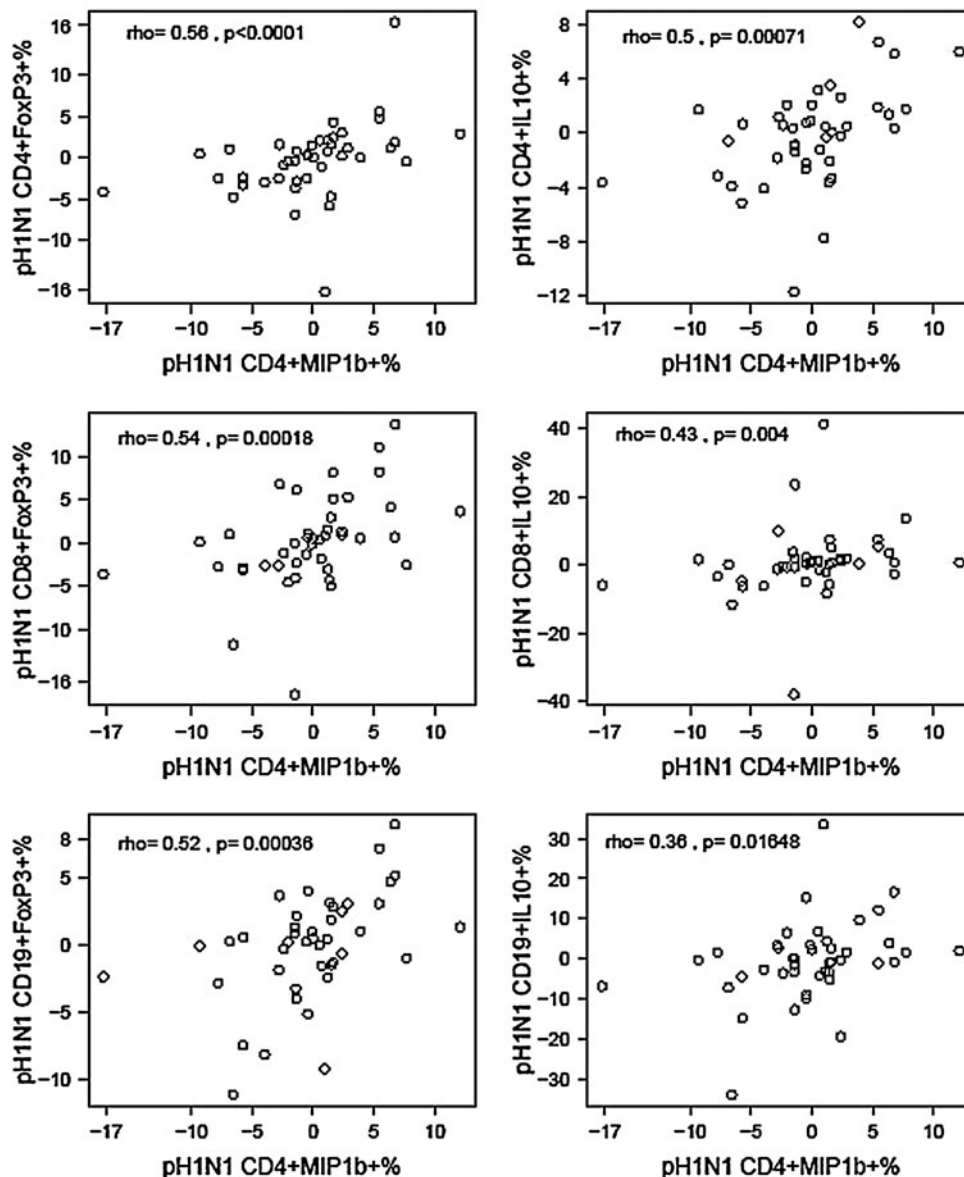
Variable (%) <sup>a,b</sup>	Median (IQR)	p value <sup>c</sup>
CD4 <sup>+</sup> FoxP3 <sup>+</sup>	0.29 (−2.50, 1.59)	0.97
CD4 <sup>+</sup> IL-10 <sup>+</sup>	0.37 (−2.08, 1.78)	0.87
CD4 <sup>+</sup> IL-10 <sup>+</sup> FoxP3 <sup>+</sup>	−0.04 (−0.34, 0.18)	0.40
CD4 <sup>+</sup> TGF- $\beta$ <sup>+</sup>	<b>−0.99 (−3.83, 0.87)</b>	<b>0.02</b>
CD4 <sup>+</sup> TGF- $\beta$ <sup>+</sup> FoxP3 <sup>+</sup>	−0.11 (−0.56, 0.27)	0.14
CD8 <sup>+</sup> FoxP3 <sup>+</sup>	0.51 (−2.59, 3.65)	0.53
CD8 <sup>+</sup> IL-10 <sup>+</sup>	0.55 (−2.23, 2.26)	0.40
CD8 <sup>+</sup> IL-10 <sup>+</sup> FoxP3 <sup>+</sup>	0.12 (−0.40, 0.82)	0.48
CD8 <sup>+</sup> TGF- $\beta$ <sup>+</sup>	−0.79 (−3.59, 1.21)	0.18
CD8 <sup>+</sup> TGF- $\beta$ <sup>+</sup> FoxP3 <sup>+</sup>	−0.15 (−0.55, 0.85)	0.98
CD19 <sup>+</sup> FoxP3 <sup>+</sup>	0.47 (−1.53, 2.54)	0.46
CD19 <sup>+</sup> IL-10 <sup>+</sup>	−0.71 (−4.13, 2.70)	0.47
CD19 <sup>+</sup> IL-10 <sup>+</sup> FoxP3 <sup>+</sup>	−0.01 (−0.16, 0.33)	0.49
CD19 <sup>+</sup> TGF- $\beta$ <sup>+</sup>	0.08 (−3.66, 1.11)	0.51
CD19 <sup>+</sup> TGF- $\beta$ <sup>+</sup> FoxP3 <sup>+</sup>	0.01 (−0.35, 0.24)	0.73

<sup>a</sup>N=45<sup>b</sup>Subsets, measured by flow cytometry, are expressed as a percentage of the parent CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> population.<sup>c</sup>Wilcoxon matched pairs signed-rank test.*Bold* represents the values that were significant at a 0.05 level.

PBMC stimulation with pH1N1 or PHA. The lack of specificity of the baseline IFN- $\gamma$  ELISpot results that predicted a good specific CMI response to pH1N1 vaccination suggested that they functioned as a measure of the overall ability of the host to mount CMI responses. The corollary of this observation is that nonspecific high ELISpot responses in HIV-infected individuals may predict their ability to mount specific CMI to vaccines and/or infections in general.

We found a marginal association of high pH1N1-CMI responses with low CD8<sup>+</sup> T cells, which is in agreement with previous reports,<sup>20</sup> but we did not find any associations with other traditional markers of HIV disease such as CD4<sup>+</sup> cell numbers or plasma HIV load. It is important to note that all women in this study received cART and a majority had high CD4<sup>+</sup> cell numbers and <1,000 HIV RNA c/ml of plasma, which leaves open the possibility that subjects with a wider range of values on these variables may exhibit an association of CD4<sup>+</sup> cell numbers and plasma HIV load with CMI responses to vaccination. It is also important to mention that in our previous studies we showed a positive association of entry CD4<sup>+</sup> T cells with the magnitude of antibody responses to this vaccine and a negative association of HIV viral load with pH1N1-specific IFN- $\gamma$  ELISpot responses after vaccination.

To determine if T cell regulation played a role in the heterogeneity of the CMI responses to pH1N1 vaccine, we investigated the relationship of pH1N1-specific Th1 and cytotoxic T cell responses to the vaccine with circulating, nonspecific Treg and Breg at baseline and with pH1N1-specific Treg and Breg after vaccination. pH1N1-specific effector T cell responses to vaccination correlated with high pH1N1-specific Treg and Breg after vaccination, suggesting that Treg and Breg were generated through a feedback mechanism as previously proposed.<sup>21</sup> High CMI responses to pH1N1 vaccine also correlated with low circulating CD19<sup>+</sup>FOXP3<sup>+</sup>% Breg at baseline, albeit not with the better characterized CD19<sup>+</sup>IL-10<sup>+</sup>% Breg subset. Nevertheless, the data suggest that a high nonspecific B cell regulatory environment may reduce CMI responses to vaccination



**FIG. 1.** Correlations between change from baseline to postdose 1 in pH1N1-specific CD4<sup>+</sup>MIP1b+% and regulatory T cell subsets. Data were derived from peripheral blood mononuclear cells (PBMC) of 52 HIV-infected pregnant women collected before and 21–28 days after the administration of a double-dose of pH1N1 IIV1. Data points represent pH1N1-specific T cell subset frequencies at postvaccination after subtraction of pre-vaccination frequencies. The coefficients of correlations and  $p$  values measured with the Spearman correlation test shown on the graphs demonstrate highly significant associations between the changes in CD4<sup>+</sup>MIP1b+% ( $x$  axes), representing Th1 responses, in response to vaccination and selected Treg- and Breg-subset% changes ( $y$  axes) after vaccination.

similarly to the effect of Breg on HIV-1-specific T cell responses of infected individuals.<sup>22</sup> In contrast, high pH1N1-specific CMI responses after vaccination correlated with high baseline circulating CD4<sup>+</sup>CD39+% and CD8<sup>+</sup>CD39+% Treg, which was surprising in view of their presumed regulatory activity. CD4<sup>+</sup>CD39<sup>+</sup> Treg are increased in HIV-infected individuals.<sup>23–25</sup>

Although CD39<sup>+</sup> Treg have not been shown to correlate with opportunistic infections or any AIDS or non-AIDS adverse events, they have been presumed to contribute to the immune suppression that characterizes HIV infection through diminution of IL-2 synthesis.<sup>24</sup> CD39 is an ectoenzyme that converts ATP to ADP/AMP. CD39 acts in concert with CD73, which converts ATP, ADP, and AMP to adenosine, which binds to the adenosine receptor and activates the T cell inhibitory pathway. Interestingly, CD73<sup>+</sup> Treg have been associated with decreased T cell activation, inflammation, and general immune preservation, while CD39<sup>+</sup> Treg have been associated with HIV immune suppression.<sup>26–28</sup>

In summary, the effect of CD39<sup>+</sup> Treg in the context of HIV infection is controversial and needs to be further investigated. Our data also need to be confirmed and mechanistically explained in future studies. As new Treg counteractive treatment modalities are being developed,<sup>29–31</sup> it is important to continue to study the relationship of Treg and Breg with CMI responses to vaccination, which may provide a framework to understand the potential for increasing responses to vaccines in HIV-infected individuals by manipulation of their Treg and Breg populations.

This study had some limitations, including the investigation of a small subset of CMI responses and Treg and Breg. In addition, we did not have a control group of HIV-uninfected pregnant women to definitively show that the heterogeneity of the responses was HIV specific. In line with the exploratory nature of the study, we did not adjust the analyses for multiple comparisons in order to capture all possible signals, and the results presented here need to be confirmed by additional studies.

TABLE 5. CORRELATION BETWEEN pH1N1-SPECIFIC CD4<sup>+</sup>MIP1 $\beta$ <sup>+</sup>% CHANGES FROM BASELINE TO POSTDOSE 1 pH1N1 VACCINE AND BASELINE CHARACTERISTICS WITH POTENTIAL PREDICTING VALUE

Characteristic <sup>a</sup>	$\rho^b$	p value	N
Race, black	-0.002	0.99	46
Ethnicity, latino	0.14	0.35	47
Type ARV regimen, HAART	0.01	0.95	49
pH1N1 HAI titers $\geq 40$	-0.04	0.79	49
Age (years)	0.09	0.52	49
CD4 count (cells/mm <sup>3</sup> )	-0.03	0.86	49
CD4 %	0.12	0.42	49
CD8 count (cells/mm <sup>3</sup> )	-0.29	0.05	46
CD8 %	-0.22	0.13	49
log <sub>10</sub> HIV-RNA (cp/ml)	-0.12	0.41	49
pH1N1 HAI titers	0.02	0.92	49
pH1N1 IFN- $\gamma$ SFC	<b>0.35</b>	<b>0.02</b>	<b>44</b>
pH1N1 granzyme B SFC	0.08	0.62	41
PHA IFN- $\gamma$ SFC	0.28	0.06	44
CD4 <sup>+</sup> CD39 <sup>+</sup> %	<b>0.37</b>	<b>0.03</b>	<b>36</b>
CD4 <sup>+</sup> HLADR <sup>+</sup> CD38 <sup>+</sup> %	0.12	0.50	36
CD4 <sup>+</sup> TGF- $\beta$ <sup>+</sup> %	0.18	0.28	36
CD8 <sup>+</sup> CD39 <sup>+</sup> %	<b>0.38</b>	<b>0.02</b>	<b>36</b>
CD8 <sup>+</sup> HLADR <sup>+</sup> CD38 <sup>+</sup> %	0.17	0.32	36
CD8 <sup>+</sup> TGF- $\beta$ <sup>+</sup> %	0.16	0.34	36
CD4 <sup>+</sup> IL-10 <sup>+</sup> %	0.27	0.10	37
CD4 <sup>+</sup> FOXP3 <sup>+</sup> %	-0.22	0.19	37
CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup> %	-0.21	0.21	37
CD8 <sup>+</sup> IL-10 <sup>+</sup> %	0.13	0.46	37
CD8 <sup>+</sup> FOXP3 <sup>+</sup> %	-0.19	0.27	37
CD8 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup> %	-0.14	0.41	37
CD19 <sup>+</sup> IL-10 <sup>+</sup> %	0.18	0.28	36
CD19 <sup>+</sup> FOXP3 <sup>+</sup> %	<b>-0.36</b>	<b>0.03</b>	<b>36</b>
CD19 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup> %	-0.11	0.53	36
CD19 <sup>+</sup> CD25 <sup>+</sup> %	0.06	0.72	36

<sup>a</sup>Subsets, measured by flow cytometry, are expressed as a percentage of the parent CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> population.

<sup>b</sup>Spearman correlation coefficients.

**Bold** represents the values that were significant at a 0.05 level.

*Italics* indicates the values that were marginally significant.

SFC, spot-forming cells.

Our findings are highly significant for the medical practice, because health care providers frequently use CD4<sup>+</sup> cell numbers and HIV load to gauge the optimal time for vaccine administration to HIV-infected individuals. Our data indicate that in HIV-infected pregnant women on cART, the predictive value of these traditional markers is reduced for CMI responses. In contrast, nonspecific CMI responses had a strong predictive value for T cell vaccine immunogenicity. If confirmed in other groups of HIV-infected individuals, this observation may suggest the value of assessing CMI function (by ELISpot or other cytokine production) as an indicator of optimal timing for vaccination.

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Participating sites and site personnel: University of Miami Pediatric/Perinatal HIV/AIDS CRS (Amanda Cotter, MD; Gwendolyn B. Scott, MD; Erika Lopez-Bertiery, MD; Safia Khan, MD); University of Puerto Rico Pediatric HIV/AIDS Research Program CRS (Irma L. Febo, MD; Carmen D. Zorrilla, MD; Vivian Tamayo-Agrait, MD; Ruth Santos, RN, MPH); University of Southern California, LA NICHHD CRS (Alice Stek, MD; Michael Neely, MD; LaShonda Spencer, MD; Andrea Kovacs, MD); Washington Hospital Center NICHHD CRS (Sara Parker, MD; Patricia Tanjutco, MD; Vanessa Emmanuel, BA; Liv Thulin); Texas Children's Hospital CRS (Shelly Buschur, RN, CNM; Mary Paul, MD; Filiz Seeborg, MD; Kathy Pitts, PhD); Chicago Children's CRS (Jessica Shore, RN; Sarah Sutton, MD); UCSD Maternal, Child, and Adolescent HIV CRS (Stephen A. Spector, MD; Andrew Hull, MD; Mary Caffery, RN, MSN; Jean Manning, RN, BSN); DUMC Pediatric CRS (Margaret Donnelly, PA; Mary Jo Hassett, RN; Elizabeth Livingston, MD; Julieta Giner, RN); Rush University Cook County Hospital, Chicago NICHHD CRS (Mariam Aziz, MD; Latania Logan, MD; Julie Schmidt, MD; Helen Cejtin, MD); The Children's Hospital of Philadelphia IMPAACT CRS (Samuel Parry, MD; Rita Leite, MD); University of South Florida, Tampa NICHHD CRS (Karen L. Bruder, MD; Gail Lewis, RN; Patricia Emmanuel, MD; Tampa General Hospital); San Juan City Hospital PR NICHHD CRS (Elvia Perez, MPH, MA; Rodrigo Diaz, MD; Dalila Guzman, RPh; Midnela Acevedo-Flores, MD); South Florida CDC, Ft. Lauderdale NICHHD CRS; Johns Hopkins University, Baltimore NCHD CRS (Allison Agwu, MD, ScM; Todd Noletto, MPH; Jennifer Chang, BS; Andi Weiss, PharmD); Tulane University, New Orleans NICHHD CRS (Chi Dola, MD; Thomas Alchediak, MD; Yvette Luster, RN; Sheila Bradford, RN); Bronx-Lebanon Hospital IMPAACT CRS (Jenny Gutierrez, MD; Mahboobullah Mirza Baig, MD; Stefan Hagmann, MD; Murli Purswani, MD); NJ Medical School CRS (Arlene D. Bardeguez, MD, MPH; Charmane Calilap-Bernardo, RN; Linda Bettica, RN); Columbia IMPAACT CRS (Andrea Jurgrau, CNP; Gina Silva, RN; Alice Higgins, RN; Marc Foca, MD); Metropolitan Hospital NICHHD CRS (Mahrukh Bamji, MD; Santa Paul, MD; Siobhan Riley, MPH; Deepali Jain, MD); Children's Hospital of Boston NICHHD CRS (Sandra K. Burchett, MD, MS; Ruth Tuomala, MD; Arlene Buck, RN; Catherine Kneut, RN, CPNP); University of Colorado, Denver NICHHD CRS (Jennifer Dunn, FNP-C; Paul Harding, MS; Kay Kinzie, FNP-C; Jenna Wallace, MSW; Supported by NIH/NCATS Colorado CTSI Grant Number

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#### Author Disclosure Statement

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Address correspondence to:

Adriana Weinberg

Mail Stop 8604

12700 East 19th Avenue, Room 11126

Aurora, Colorado 80045

E-mail: [adriana.weinberg@ucdenver.edu](mailto:adriana.weinberg@ucdenver.edu)